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Abstract

Transmissible spongiform encephalopathies are commonly propagated by extracerebral inoculation of the infectious agent. Indirect evidence suggests that entry into the central nervous system occurs via the peripheral nervous system. Here we have investigated the role of the sympathetic nervous system in prion neuroinvasion. Following intraperitoneal prion inoculation, chemical or immunological sympathectomy delayed or prevented scrapie. Prion titers in spinal cords were drastically reduced at early time points after inoculation. Instead, keratin 14-NGF transgenic mice, whose lymphoid organs are hyperinnervated by sympathetic nerves, showed reduction in scrapie incubation time and, unexpectedly, much higher titers of prion infectivity in spleens. We conclude that sympathetic innervation of lymphoid organs is rate limiting for prion neuroinvasion and that splenic sympathetic nerves may act as extracerebral prion reservoirs.
Sympathetic Innervation of Lymphoreticular Organs Is Rate Limiting for Prion Neuroinvasion

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Summary
Transmissible spongiform encephalopathies are commonly propagated by extracerebral inoculation of the infectious agent. Indirect evidence suggests that entry into the central nervous system occurs via the peripheral nervous system. Here we have investigated the role of the sympathetic nervous system in prion neuroinvasion. Following intraperitoneal prion inoculation, chemical or immunological sympathectomy delayed or prevented scrapie. Prion titers in spinal cords were drastically reduced at early time points after inoculation. Instead, keratin 14-NGF transgenic mice, whose lymphoid organs are hyperinnervated by sympathetic nerves, showed reduction in scrapie incubation time and, unexpectedly, much higher titers of prion infectivity in spleens. We conclude that sympathetic innervation of lymphoid organs is rate limiting for prion neuroinvasion and that splenic sympathetic nerves may act as extracerebral prion reservoirs.

Introduction
Prion diseases are fatal neurodegenerative diseases of humans and animals. The infectious agent is termed prion (Aguzzi and Weissmann, 1997) and is thought to be identical with PrPSc, a protease-resistant conformer of the cellular protein PrPC (Prusiner, 1982). Although prions are most efficiently propagated through intracerebral inoculation, peripheral infection is the natural route of transmission in most prion diseases. Kuru, BSE, and the new variant of Creutzfeldt-Jakob disease are caused by oral uptake of the infectious agent, while parenteral administration of growth hormone and gonadotropins has resulted in iatrogenic Creutzfeldt-Jakob disease (Glatzel and Aguzzi, 2000a).

The pathways by which prions invade the central nervous system (CNS) are only partially understood. A large body of evidence indicates that both the lymphoreticular system (LRS) and peripheral nerves are involved in scrapie neuroinvasion (Klein et al., 1997; Lasmezas et al., 1996; Race et al., 2000). The mode of transport of infectivity in peripheral nerves has not been determined yet. Recent studies point to the possibility that the infectious agent utilizes mechanisms distinct from conventional axonal transport (Glatzel and Aguzzi, 2000b; Hainfellner and Budka, 1999) and may involve PrPSc-specific binding proteins (Fischer et al., 2000). Upon intraperitoneal (i.p.) inoculation with prions, pathological lesions and prion replication appear first in the thoracic spinal cord in the segments corresponding to the entry site of the splanchnic nerves that belong to the sympathetic nervous system (SNS) (Beekes et al., 1996; Cole and Kimberlin, 1985). Furthermore, prions accumulate in sympathetic ganglia (McBride and Beekes, 1999). Considering that lymphoid organs are predominantly innervated by sympathetic nerve fibers, the SNS seems to be a prime target for transport and perhaps also for replication of the prion agent (Felten and Felten, 1988).

Lymphoid organs, including the spleen, are early sites of accumulation and replication of the agent following i.p. inoculation (Eklund et al., 1967; Kimberlin and Walker, 1989). Ablation of B cells suppresses prion neuroinvasion (Klein et al., 1997, 1998), probably because B cells transmit a lymphotxin-β-dependent signal to follicular dendritic cells (FDCs) (Montrasio et al., 2000), which are a prominent site of PrPSc deposition (Kitamoto et al., 1991).

In order to study the role of the SNS in neuroinvasion, we sympathectomized mice using three different protocols. Permanent sympathectomy was performed by injecting 6-hydroxydopamine (6-OHDA), or anti-nerve-growth factor antibodies (anti-NGF Ab), into newborn mice. Transient sympathectomy was achieved by administering 6-OHDA over a period of 7 weeks to 8- to 10-week-old mice. All of these methods cause efficient and protracted sympathectomy (Picklo, 1997). We found that permanent and transient sympathectomy significantly delayed, or even prevented, development of terminal scrapie upon i.p. inoculation of the infectious agent.

The role of the SNS was further investigated with transgenic mice overexpressing nerve growth factor in peripheral nerves. Prion infectivity in these mice was significantly reduced when compared to nontransgenic control mice. Surprisingly, hyperinnervated spleens contain >100 fold more infectivity, suggesting that splenic nerve endings may act as extracerebral prion reservoirs.

Results
Efficient and Specific Sympathectomy Using 6-OHDA or a Monoclonal Anti-NGF Antibody
Selective sympathectomy was performed by administering 6-OHDA or anti-NGF Ab to wild-type mice. All methods induced significant, but not complete depletion of sympathetic nerves as assessed by Western blot analysis (Figure 1A) and by immunohistochemical stains of spleens (Figure 1B) for tyrosine hydroxylase (TH), a marker of sympathetic nerves innervating lymphoid organs. There was no significant difference in the distribution and amount of FDCs between sympathectomized
Figure 1. Effects of 6-OHDA-Induced Denervation and of K14NGF-Induced Hyperinnervation on Mouse Spleens
(A) Western blot analysis of TH in spleens of adult 6-OHDA-treated mice, K14NGF transgenic mice, and controls. The two main TH bands, visible at ca. 55 kDa, were stronger in older mice (after four injections and later), consistent with the increase in sympathetic innervation with age. Lower panel: quantification of TH content by chemiluminescence scanning. Bars represent the ratio between signal intensity of the TH band and that of the corresponding β-actin bands (arbitrary units).

(B) Confocal immunofluorescence analysis of control (NaCl-treated), denervated (6-OHDA-treated), and hyperinnervated K14NGF spleens. Sympathetic nerves (displayed in black) are visualized with anti-TH antibodies. Sympathetic innervation was mainly localized around vessels (arrows), although some nerve fibers protrude in the splenic parenchyma (asterisk). 6-OHDA-treated spleens contained only single occasional fibers, while hyperinnervated spleens showed abundant nerve fibers around vessels and in the splenic parenchyma.

(C) Spleen sections of NaCl-injected and 6-OHDA-injected mice immunostained with the FDC-specific antibody FDC-M1 showed no difference in the topography and size of FDCs as assessed by morphometry (right-most panel). Bars represent the area occupied by the FDC signal (in 1000 μm² per ten high-power fields).

(n = 6) and control mice (n = 3) as analyzed by immunohistochemical staining with antibody FDC-M1 to FDCs (Figure 1C). To rule out any putative impairment of immune cells in sympathectomized mice, blood and spleens before and after 6-OHDA injections were analyzed for the presence and total numbers of blood cells utilizing a hemocytometer and fluorescence-activated cell sorting (FACS). There was no change in blood and splenic CD4+ and CD8+ T lymphocytes (data not shown). We detected a slight increase in CD11b+ leukocytes and B220/CD45R+ B lymphocytes in peripheral blood and spleens. After the first injection of 6-OHDA, FACS analysis evidenced a higher amount of leukocytes with a relative decrease in B lymphocytes within a measured population of 10,000 cells (Figure 2). Total numbers of lymphocytes and leukocytes in peripheral blood, as determined by hemocytometer, showed mild increase in CD11b+ cells and a minor increase in lymphocytes (data not shown).

Sympathectomy Delays Development of Scrapie Following Peripheral Prion Administration
Sympathectomized mice display significant prolongation of incubation times until terminal scrapie when challenged with various doses of prions. The effect was more pronounced when permanent denervation was performed by administering 6-OHDA or anti-NGFAb neonatally (Figure 3B). In these groups, the mean incubation times until the onset of terminal scrapie were 244 ± 20 days (10^6 LD50 units, n = 7) for 6-OHDA-treated mice and 254 ± 18 days (10^4 LD50 units, n = 6) for anti-NGFAb-treated mice. One mouse was sacrificed at day 220 because of wasting and did not show histopathological signs of scrapie (Figures 4I and 4J). Untreated mice developed terminal scrapie disease after 189 ± 4 days (10^6 LD50 units, n = 4) and 214 ± 1 days (10^4 LD50 units, n = 4) (Figure 3B).

Incubation times for mice denervated as adults were 220 ± 4 days (10^6 LD50 units, n = 7) and 279 ± 25 days (10^3 LD50 units, n = 5) (Figure 3A). One mouse had to be taken at day 292 because of wasting and did not show histopathological signs of scrapie or accumulation of PrPSc in brain or spleen (Figure 5A). The respective control groups developed terminal scrapie disease after 237 ± 3 days (10^6 LD50 units, n = 5) and 184 ± 9 days (10^5 LD50 units, n = 5) (Figure 3A). None of the mock-inoculated control mice developed neurological disease. These mice were sacrificed at day 288 (n = 4 for
Figure 2. FACS Analysis of Peripheral Blood and Spleen Cells at Various Time Points after Injection of 6-OHDA or of Vector

Density plots of the relative frequencies (indicated in percentages of 10,000 cells) of B220/CD45R<sup>+</sup> B lymphocytes (vertical axis) and CD11b<sup>+</sup> leukocytes (horizontal axis). A slight rise in CD11b<sup>+</sup> cells and a relative decrease in B220/CD45R<sup>+</sup> B lymphocytes were evident after the first injection of 6-OHDA in peripheral blood and in spleen, yet both populations returned to normal levels after additional injections.

the anti-NGF Ab group) or died of unrelated causes after day 360 (n = 5 for the 6-OHDA-treated group).

It has been reported that peripheral administration of 6-OHDA or of anti-NGF Ab does not affect the synthesis or metabolism of TH in the CNS (Picklo, 1997). To determine whether the same is true for the expression and processing of PrPC, we performed quantitative Western blot analyses for PrPC<sup>C</sup> on CNS tissue samples of dener-vated and control mice. There was no alteration in the amount of PrPC<sup>C</sup> or in the glycosylation pattern of PrPC<sup>C</sup> between the two groups (Figure 6). This indicates that the delay in incubation times of sympathectomized mice is not due to downregulation of PrPC<sup>C</sup> in the CNS.

No Evidence for Neuroinvasion via Vagal Nerve in Sympathectomized and in Control Mice

Upon oral challenge, prions appear to spread via the vagal nerve (Baldauf et al., 1997; Beekes et al., 1998). In order to investigate the utilization of this route, we analyzed the dorsal motor nucleus of the vagal nerve in sympathectomized mice. However, the degree of gliosis and spongiosis did not vary between scrapie-sick de-ervated (n = 8) and nondener-vated (n = 6), or between vagal and nonvagal brain stem nuclei of dener-vated mice (Figure 7). Therefore, we could not find any evidence that vagal spread of prions substitutes for symp-pathetic spread in sympathectomized mice.

Reduced Scrapie Pathology and Prion Replication in Spinal Cords of Sympathectomized Mice 90 Days after Prion Administration

To confirm that elimination of the SNS affects transport from lymphoid organs to the CNS, we studied the mor-phology and prion titer of spinal cords 90 days after i.p. inoculation with scrapie prions. We focused on thoracic segments 7–9 because it was shown that the first pathological changes and replication of infectivity occur in this region (Cole and Kimberlin, 1985). Transiently sympathectomized mice (n = 6) showed significantly less gliosis and spongiosis when compared to the control mice (n = 4) (Figures 4A and 4B). Surprisingly, no PrP<sup>Sc</sup> could be detected in any of the sympathectomized or control mice by histoblot and Western blot (data not shown). We determined prion titers in two thoracic spinal cord segments of treated and untreated mice using the incubation time assay (Prusiner et al., 1982) calibrated
Figure 3. Survival Plots Displaying the Incubation Time (Days) until Development of Terminal Scrapie in C57BL/6 Mice Inoculated i.p. with Prions and of Tau20 Indicator Mice Inoculated i.c. with Spinal Cord and Spleen Samples

(A–C) Mice injected with 6-OHDA in their adulthood (A), as well as mice injected neonatally with anti-NGF antibodies or with 6-OHDA (B) developed terminal scrapie significantly later than their respective controls. Instead, K14NGF mice, which have hyperinnervated spleens, developed terminal scrapie earlier than matched wild-type siblings of the same genetic background (C). The size of inoculum is expressed in logLD50 units.

(D) Prion titers (right bar) as calculated by the incubation time (left bar) method. Tested samples (as indicated) were taken at different time points (upper horizontal bar). Diamonds indicate individual mice. Small horizontal bars indicate average incubation time. Spinal cords from 6-OHDA-treated mice showed very low or undetectable levels of prion infectivity. Spleens of hyperinnervated K14NGF transgenic mice displayed titers of infectivity that were at least two logs higher than those of wild-type mice.
Figure 4. Histopathological Findings in Spinal Cords (Level 7–9) and Hippocampi of Mice Sacrificed after Prion Inoculation

Prominent spongiosis (C, E, and G) and gliosis (B, D, F, and H) are visible in spinal cords (left column) and hippocampi (all other columns) of terminally sick mice. Spinal cords of 6-OHDA-injected mice showed no detectable gliosis (A). One of the neonatally anti-NGFαb-injected mice showed no spongiosis (I) nor gliosis nor scrapie symptoms when sacrificed 288 days after inoculation (J), potentially indicating complete protection from scrapie.

with tgα20 indicator mice (Brandner et al., 1996). As expected, prion titers of spinal cords from untreated mice were high (4.1 logLD₅₀ mg⁻¹ and 4.9 logLD₅₀ mg⁻¹), whereas spinal cords from sympathectomized mice did not contain detectable infectious prions (<0 logLD₅₀ mg⁻¹) (Figure 3D).

Incubation Times of Sympathectomized and Control Mice Are Unchanged Following Intracerebral Inoculation with Prions

In order to rule out any unspecific effects of 6-OHDA on the development of scrapie, we inoculated transiently 6-OHDA-treated and control mice with prions intracerebrally. All inoculated mice developed scrapie symptoms as expected. There was no significant difference in the incubation time until development of terminal scrapie symptoms. Incubation times for denervated mice were 175 ± 2 days and 182 ± 1 days for controls (both 3 logLD₅₀ units, n = 3).

K14NGF Mice Show Shortened Scrapie Incubation Times and High Titers of Prion Infectivity in Spleens

If sympathetic innervation is a rate-limiting determinant for prion neuroinvasion, hyperinnervated mice should show a decrease in incubation times until the onset of scrapie disease. To test this prediction, we analyzed scrapie pathogenesis in transgenic mice overexpressing NGF under transcriptional control of the keratin 14 promoter, which develop massive hyperinnervation of lymphatic organs (Carlson et al., 1995) (Figures 1A and 1B). Incubation times until development of terminal scrapie disease after i.p. inoculation were 21 days shorter (209 ± 15 days, n = 12) for K14NGF mice than for wild-type mice (230 ± 9 days, n = 6) (Figure 3C). Histoblot analyses of spinal cords showed considerably more accumulation of PrPSc in spleens of K14NGF mice (Figures 5A and 5B). Surprisingly, splenic prion titers of hyperinnervated mice were much higher than prion titers of wild-type mice (4.7, 4.9, 4.9 logLD₅₀ × mg⁻¹ for K14NGF, 2.7, 2.6 logLD₅₀ × mg⁻¹ for controls) (Figure 3D).

K14NGF Mice Do Not Show Immunological Alterations

Since the immune system plays a key role in prion pathogenesis, we investigated salient immune parameters in K14NGF mice. FACS analysis of spleen and blood using a range of monoclonal antibodies directed against B cells, T cells, their subsets, and macrophages did not reveal any immune system alterations in K14NGF mice (data not shown). These results are consistent with previous studies in which functional immunological assays did not reveal differences between transgenic and wild-type mice (Carlson et al., 1997).

Prion Titers in Spinal Cords of K14NGF Mice

Four K14NGF and three wild-type mice were sacrificed 50 days after i.p. inoculation. Western blot and histoblot analyses of spinal cords did not show accumulation of PrPSc in any of the examined mice (Figure 6). Next we determined prion titers in thoracic spinal cords (segments 7–9) (n = 3 for K14NGF, n = 2 for controls). One of the spinal cords taken from a K14NGF transgenic mouse showed a high prion titer (4.7 logLD₅₀ × mg⁻¹), whereas titers from the other transmitted spinal cords displayed lower prion titers (3.8 logLD₅₀ × mg⁻¹ for K14NGF and 3.2 logLD₅₀ × mg⁻¹ for control). In one spinal cord of the hyperinnervated and control mice, the prion titer was below detection limit for the bioassay.

Discussion

Evidence that Sympathetic Innervation of Lymphoreticular Organs Is Involved in Neuroinvasion

Peripheral challenge of mice with prions results in accumulation of the infectious agent in organs of the lymphoid system at very early time points (Eklund et al., 1967; Lasmezas et al., 1996). Prion transfer from the
Figure 5. Accumulation of PrP\textsuperscript{Sc} in Brains and Spleens of Mice Inoculated i.p. with Prions

(A) Histoblots showing immunoreactive PrP\textsuperscript{C} in brains (left) and spleens (right) before (1\textsuperscript{st} and 4\textsuperscript{th} column) and after digestion (2\textsuperscript{nd}, 3\textsuperscript{rd}, 5\textsuperscript{th}, and 6\textsuperscript{th} column) with increasing concentrations of proteinase K. Mock-inoculated mice (6\textsuperscript{th} row) show proteinase K-sensitive PrP\textsuperscript{C} but no proteinase K-resistant PrP\textsuperscript{Sc} in brains and spleens. One of the 6-OHDA-injected mice (euthanized at 294 days because of progressive ascites) did not show any PrP\textsuperscript{Sc} in brain or spleen (1\textsuperscript{st} row). Terminally sick mice showed proteinase K-sensitive PrP\textsuperscript{C} and proteinase K-resistant PrP\textsuperscript{Sc} in brains and spleens. However, accumulation of PrP\textsuperscript{Sc} in spleens of 6-OHDA- or anti-NGF Ab-injected mice (rows 2 and 3) was not as strong as in wild-type mice (row 4). The fifth row shows the pattern of PrP\textsuperscript{Sc} accumulation in a terminally sick K14NGF transgenic mouse. Note the intense accumulation of PrP\textsuperscript{Sc} in this mouse compared to the wild-type control.

(B) Higher magnification of tissues showed in (A), demonstrating the difference in spleen PrP immunoreactivity after proteinase K digest of terminally sick wild-type (left side) and hyperinnervated K14NGF transgenic mice (right side). This finding is paralleled by higher prion titers in spleens of hyperinnervated mice (see Figure 3D).

(C) Western blot detection of PrP\textsuperscript{C} and PrP\textsuperscript{Sc}. Left blot, brain; right blot, spleen. Proteinase K digest as indicated above each lane (+ or −). For controls, we analyzed terminally sick C57BL/6 mice (pos. cont.), mock injected C57BL/6 mice (neg. cont.), and Prnp knockout mice (Prnp\textsuperscript{o/o}). PrP\textsuperscript{Sc} was detectable in brains and spleens of all mice that presented with scrapie symptoms. The particularly strong signal in one brain of a 6-OHDA-treated mouse (5\textsuperscript{th} lane from left) is most likely the result of natural variations in the amount of proteinase-resistant prion protein in terminally sick mice.
laboratory to the CNS occurs most likely along peripheral nerves in a PrP<sup>C</sup>-dependent fashion (Bla¨ttler et al., 1997; Glatzel and Aguzzi, 2000b; Race et al., 2000). Studies focusing on the temporal and spatial dynamics of neuroinvasion have suggested that the autonomic nervous system might be responsible for transport from lymphoid organs to the CNS (Beekes et al., 1998; Clarke and Kimberlin, 1984; Cole and Kimberlin, 1985; McBride and Beekes, 1999). The innervation pattern of lymphoid organs is mainly sympathetic (Felten and Felten, 1988).

To clarify the role of the SNS in neuroinvasion of prions, we depleted or enhanced sympathetic innervation of lymphoid organs using various regimens. All three sympathectomy protocols chosen, i.e., injection of 6-OHDA in adult or in newborn mice or of anti-NGF antibodies in newborn mice, were shown by histological and biochemical methods to reduce the amount of noradrenergic nerve fibers substantially. Sympathetic denervation significantly delayed the development of scrapie in all experimental designs. In one mouse denervated by 6-OHDA injection and in one mouse of the anti-NGFAb group, treatment blocked prion neuroinvasion completely and prevented all clinical signs of scrapie. A detailed temporal breakup of infectivity titers in spinal cords indicates that sympathectomy inhibited the transport of prions from lymphatic organs to the thoracic spinal cord, which is the entry site of sympathetic nerves to the CNS (Figure 3D).

We attempted to correlate these findings with quantitative measurements of PrP<sup>Sc</sup> in spinal cords but were unable to detect PrP<sup>Sc</sup> at 50–90 days post inoculation. This is probably because biochemical methods are several orders of magnitude less sensitive than prion infectivity bioassays.

The importance of the SNS was further underscored by the time to onset of clinical scrapie in transgenic mice with sympathetic hyperinnervation of immune organs. Hyperinnervated mice developed scrapie symptoms significantly earlier than their nontransgenic controls.

**Figure 6. Accumulation of PrP<sup>Sc</sup> in Brains and Spinal Cords of Control and Hyperinnervated Mice**

Western blot analysis of PrP in brains of 6-OHDA-treated, control mice and in spinal cords (segments Th7–9) of K14NGF and of wild-type mice euthanized 50 days after prion administration. There were no alterations in the extent or in the glycosylation pattern of brain PrPC. No accumulation of PrP<sup>Sc</sup> was detectable in spinal cords of K14NGF and wild-type mice at this early time point. Terminally sick C57Bl/6 mice, mock injected C57Bl/6 mice, and Prnp<sup>o/o</sup> knockout mice (Prnp<sup>−/−</sup>) were used as controls. Plus and minus signs above each lane indicate whether proteinase K digest was performed.

**Figure 7. Histological Analysis of Brain Stems in Sympathectomized Mice**

(A–D) Dorsal motor nuclei of the vagal nerve (n.d.v., indicated as red square in [F]). (E) Nucleus centralis medullae oblongatae (n.m.o., indicated as blue square in [F]). All mice were terminally scrapie-sick with the exception of (B). There is neither a difference in the degree of gliosis between denervated (C and D) and nondenervated mice (A), nor between vagal and nonvagal brain stem nuclei of denervated mice (C–E). All sections were immunostained with a GFAP antiserum.
the immune system. FACS analysis and blood cell counts of B, T cells and leukocytes showed an initial phase of mild activation, but soon returned to normal after sympathetic denervation. We attribute this activation characterized by a slight increase in total numbers of leukocytes and lymphocytes to an unspecific acute response induced by 6-OHDA. No alteration in lymphocyte subpopulations was detected in spleens at any time point investigated. Results from experimental models of sympathetic denervation have been conflicting, showing enhancement (Madden et al., 1994; Kruszew ska et al., 1995) or suppression (Kohm and Sanders, 1999) of the immune system. Our findings are in line with a possibly enhanced immune response, albeit modest and transient.

A wealth of evidence points to mature FDCs as the sites of prion colonization and replication in lymphoid organs (Kitamoto et al., 1991; Montrasio et al., 2000). Therefore, we decided to quantify the amount of FDCs in sympathectomized and control mice. We did not detect any significant differences in the content of FDCs of treated and untreated mice, which is consistent with findings from recent experiments (Kohm and Sanders, 1999) and negates the possibility that the effects observed may be due to modulation of FDC microanatomy.

**Sympathetic Nerves and the Current Models of Prion Neuroinvasion**

Transport of prions from spleen to spinal cord was studied by determining infective titer of thoracic spinal cords and spleens 50 days after i.p. injection of prions. Only one out of four spinal cords of K14NGF mice showed a high prion titer, whereas the titers of the other transgenic spinal cords were comparable to the controls. This supports the view that elimination of sympathetic nerves blocks entry into the CNS, while overexpression of sympathetic nerves does not increase the velocity of transport in the peripheral nervous system. Instead hyperinnervation may augment the development of clinical scrapie by facilitating replication of infectious prions in the spleen, since prion titers in hyperinnervated spleens were at least two logs higher than prion titers in control spleens.

In the last several years, we have elaborated a model that predicts prion neuroinvasion to consist of two distinct phases (Aguzzi et al., 2001). In the first phase, widespread colonization of lymphoreticular organs is achieved by mechanisms that depend on B-lymphocytes (Klein et al., 1997, 1998), follicular dendritic cells (Montrasio et al., 2000), and complement factors (Klein et al., 2001). The second phase has long been suspected to involve peripheral nerves, and possibly the SNS, and may depend on expression of PrPsc by these nerves (Glatzel and Aguzzi, 2000b).

While this study implies the SNS as a major component of the second phase of prion neuroinvasion, many details remain to be elucidated. In particular, it is not known whether prions can be transferred directly from FDCs to sympathetic endings, or whether additional cell types are involved. The latter possibility is particularly enticing since FDCs have not been shown to entertain physical contact with SNS terminals.

Moreover, it is unclear how prions are actually transported within peripheral nerves. Axonal and nonaxonal transport mechanisms may be involved, and nonneuronal cells (such as Schwann cells) may play a role. Within the framework of the protein-only hypothesis, one may hypothesize a “domino” mechanism, by which incoming PrPsc converts resident PrPc on the axolemmal surface, thereby propagating spatially the infection. While speculative, this model is attractive since it may accommodate the finding that the velocity of neural prion spread is extremely slow (Kimberlin et al., 1983) and may not follow the canonical mechanisms of fast axonal transport. Indeed, recent studies may favor a nonaxonal transport mechanism that results in periaxonal deposition of PrPsc (Glatzel and Aguzzi, 2000b; Hainfellner and Budka, 1999).

Our results are in agreement with previous studies showing that following peripheral administration of prions, the agent accumulates in sympathetic ganglia (McBride and Beekes, 1999), and the first pathological lesions are observed in the gray matter of midthoracic spinal cords. This corresponds to the entry site of the splanchnic nerves in the CNS (Cole and Kimberlin, 1985).

**Sympathetic Neuroinvasion and Prophylaxis of Prion Diseases**

The fact that denervated mice eventually developed scrapie may be due to (1) an alternative, low-efficiency route of entry that may become uncovered by the absence of sympathetic fibers or (2) incomplete sympathectomy. Entry through the vagal nerve has been proposed in studies of the dynamics of vacuolation following oral and i.p. challenge with prions (Baldauf et al., 1997; Beekes et al., 1998). We therefore examined vacuolation and gliosis in brain stems of denervated and nondenervated mice. There was no evidence for transport along the vagal nerve in sympathectomized mice, since vagal nuclei were affected similarly to other regions of the brain stem unrelated to the vagal system. This supports the hypothesis that delayed neuroinvasion in denervated mice may occur because of residual innervation of lymphoreticular organs.

The surprising finding that infectious titers in hyperinnervated spleens are at least two logs higher and show enhanced PrPsc accumulations in histoblots compared to control mice suggests that sympathetic nerves, besides being involved in the transport of prions, may also accumulate and replicate prions in lymphatic organs, as hypothesized earlier (Clarke and Kimberlin, 1984).

The identification of the SNS as a rate-limiting component in prion neuroinvasion also has a practical fallout: it suggests that pharmacological or genetic (Glatzel et al., 2000) manipulations of the SNS could be useful as a novel therapeutical approach to delaying or preventing neuroinvasion.

**Experimental Procedures**

**Chemical Sympathectomy**

For transient sympathectomy, female 8-week-old C57BL/6 mice were injected i.p. with 6-OHDA (Sigma, 220 mg/g mouse weight) weekly in 0.8% NaCl containing 1 × 10−4 M ascorbate as antioxidant. Mice received seven weekly injections of 6-OHDA, while control mice received ascorbate injections at the same time points. Mice in the experimental groups were injected with infectious prions or...
with mock inoculum 3 days after the first administration of 6-OHDA or ascorbate. For permanent sympathectomy, newborn C57BL/6 mice were injected with 6-OHDA (Sigma; 50 mg/g mouse weight) on postnatal days 1–4, while control mice received ascorbate injections. Only female mice were included in the further study. At 8 weeks of age, mice in the experimental groups were injected i.p. with prions or with mock inoculum. A total of 33 mice was included in the control group to study the degree of sympathetic denervation and its effects on the immune system. These mice were injected with 6-OHDA (n = 21) or NaCl (n = 12) at the same time points as the experimental mice. Spleens and blood were assayed by FACS analysis, by Western blots for TH, and by immunostains for TH, FDCs, and B and T cell markers (see below).

Inoculation of Mice
For all inoculations, we used dilutions of brain homogenate prepared as described from mice infected with the Rocky Mountain Laboratory scrapie prions (RML, passage 4.1) (Büeler et al., 1993). A total of 66 mice were inoculated i.p. using 26G needles and 200 μl syringes with dilutions of the brain homogenate (in PBS containing 5% bovine serum albumin [BSA], 100 μl inoculation volume). Six mice were intracerebrally injected with the same inoculum using a similar needle and syringe (30 μl inoculation volume). A total of nine mice were injected i.p. with mock inoculum consisting of brain homogenate prepared similarly from uninfected mice. All mice were of the C57BL/6 inbred strain, except for K14NGF transgenic mice and their controls, which were C57BL/6-C3H hybrids.

Histological Studies
Brain, spleen, and spinal cord from selected mice were fixed with 4% buffered formalin, inactivated 1 hr with 98% formic acid, and embedded in paraffin. Sections were cut to a thickness of 5 μm. Selected sections were cut in stages of 100 μm until the dorsal motor nucleus of the vagal nerve could be identified. For cryosectioning, tissues were snap-frozen in cryoprotectant compound and cut with a cryostat to a thickness of 6 μm. Floating sections were prepared at immunospecific antibodies for TH (see below). Gliosis (a nonspecific but early indicator of brain damage) was visualized by the presence of large immunostained reactive astrocytes. In addition, we performed immunostainings for TH. After blocking in 4% goat serum, floating sections were incubated overnight with a polyclonal anti-TH antibody (1:5000, Chemicon), washed, and incubated with a biotinylated secondary antibody (1:5000 goat anti-rabbit, Vector). Visualization was accomplished with the avidin-biotin method using nickel-enhanced DAB. For immunofluorescence, FITC- or rhodamine-labeled secondary antibodies were used (Sigma, Molecular Probes). To detect FDCs, we used monoclonal antibody FDC-M1 (1:300 clone 4C11). Sections were incubated with a secondary antibody (CalTag) and labeling visualized by the alkaline phosphate method after incubation with a tertiary antibody (alkaline phosphate-conjugated donkey anti-goat) (Jackson Immuno Research Laboratories). Quantification of FDCs was accomplished by measuring the area taken up by FDC signal in five representative follicles/spleen using a digital camera and analySIS software (SIS, Münster, Germany).

Infectivity Bioassays
Thoracic spinal cord (segments 7–9) and spleen homogenates (10% in 0.32 M sucrose) were prepared from infected animals by homoge-

nizing tissues using a pellet mixer and by sonicating samples for 5 min with a Branson 450 sonifier at a constant output power of 400W. 30 μl (diluted 1:10 in PBS and 1% BSA) was administered i.c. to groups of three or four fga20 (Fischer et al., 1996) mice for each sample. Incubation time until development of terminal scrapie sickness was determined and infectivity titers were calculated for 1 mg of inoculated tissue using the relationship y = −11.45 − 0.088x, where y is LD50 and x is the incubation time (days) to terminal disease (Brandner et al., 1996).

Western Blot Analysis
10% (v/v) homogenates of thoracic spinal cord (segments 7–9), brain, or spleen were prepared as described (Büeler et al., 1993) and, where indicated, digested with 20 μg/ml proteinase K (PK) for 30 min at 37°C. 50 (for TH detection and brains) or 100 (for spleens) μg of total protein was electrophoresed through 12%-SDS polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were probed with monoclonal antibody 6H4 (for brain, 1:5000, Prionics, Switzerland) or 1B3 (for spleen, 1:5000) (Farquhar et al., 1996) to mouse PrP, or with a monoclonal antibody to TH (1:5000, Sigma clone TH-2) and developed by enhanced chemiluminescence (Amersham). Quantification of TH expression was accomplished by scanning membranes with a Kodak image station 440. The signal intensities produced by spleens from control animals were measured and compared to the signal intensity of spleens from sympathomectomized mice using 1D image analysis software (Kodak).

Histoblots
Histoblots were performed according to Taraboulos et al. (1992). Frozen sections were mounted on uncoated glass slides and immediately pressed on a nitrocellulose membrane wetted in lysio buffer. Membranes were air dried for at least 24 hr. For detection, they were rehydrated in Tris-buffered saline-Tween, and limited proteolysis was performed using proteinase K concentrations of 50 and 100 μg/ml at 37°C for 4 hr. Blots were then denatured in 3 M guanidinium thiocyanate for 10 min and blocked for 1 hr in 5% non-fat milk serum. Incubation with primary antibody XN was carried out at a dilution of 1:2000 in 1% non-fat milk serum at RT for 1 hr. Detection was accomplished with an alkaline-phosphatase conjugated goat-anti-mouse antibody (1:2000). Visualization was achieved with ni troblue-tetrazolium and bromo-chloro-indolyl-phosphate according to the protocols of the supplier.

FACS Analysis and Cell Counts
Blood samples were obtained from the retrobulbar plexus of ether-anesthetized mice, and cell blood counts were determined in a hemocytometer (Advia, Boehringer Mannheim, Germany). Single-cell suspensions of spleen and blood samples, respectively, were prepared at 4°C in buffer solution (PBS containing 2% FCS and 0.2% NaN3) and stained with the following antibodies: fluorescein isothiocyanate (FITC)-labeled anti-CD11b, phycoerythrin (PE), or FITC-labeled anti-CD45R/B220, FITC-labeled anti-CD4, PE-labeled anti-CD8b, and PE-labeled anti-CD90.2 (Thy1.2) (all Pharmingen). After lysis of erythrocytes with FACS lysis solution (Becton Dickin son, San Jose, California) and washing, cell suspensions were analyzed on a FACS Calibur (Becton Dickinson). Viable cells were gated by forward and side scatter of light. Data were acquired with CellQuest software (Becton Dickinson); analyses were performed with WinMDI software.

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